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Clonal reproduction shapes evolution in the lizard malaria parasite *Plasmodium floridense*

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The preponderant clonal evolution hypothesis (PCE) predicts that frequent clonal reproduction (sex between two clones) in many pathogens capable of sexual recombination results in strong linkage disequilibrium and the presence of discrete genetic subdivisions characterized by occasional gene flow. We expand on the PCE and predict that higher rates of clonal reproduction will result in: (1) morphologically cryptic species that exhibit (2) low within-species variation and (3) recent between-species divergence. We tested these predictions in the Caribbean lizard malaria parasite *Plasmodium floridense* using 63 single-infection samples in lizards collected from across the parasite's range, and sequenced them at two mitochondrial, one apicoplast, and five nuclear genes. We identified 11 provisionally cryptic species within *P. floridense*, each of which exhibits low intraspecific variation and recent divergence times between species (some diverged approximately 110,000 years ago). Our results are consistent with the hypothesis that clonal reproduction can profoundly affect diversification of species capable of sexual recombination, and suggest that clonal reproduction may have led to a large number of unrecognized pathogen species. The factors that may influence the rates of clonal reproduction among pathogens are unclear, and we discuss how prevalence and virulence may relate to clonal reproduction.

KEY WORDS: **Cryptic species, genetic variation, Haemosporidia, preponderant clonal evolution, recent speciation.**

Clonal reproduction, or uniparental propagation, in organisms that are capable of sexual recombination has the potential to strongly impact the evolution of a large number of pathogens (Vrijenhoek 1979; Tibayrenc et al. 1990; Eisen and Schall 2000; Tibayrenc and Ayala 2002, 2012, 2014). The preponderant clonal evolution hypothesis (PCE) predicts that lineages undergoing clonal reproduction will exhibit strong linkage disequilibrium (LD) and "near-clading," or the emergence of discrete genetic lineages that appear to be stable across space and time (Tibayrenc and Ayala 2012, 2014). Near clades are not considered true clades because sexual recombination is expected to occur on occasion between near-clades, rendering them nonmonophyletic (Tibayrenc and Ayala 2012, 2014). Pathogens from across the tree of life exhibit patterns of LD and near-clading that are consistent with the PCE, including viruses (e.g., Ebola virus, dengue virus), bacteria (e.g., *Neisseria meningitidis*, *Escherichia coli*), fungi (e.g., *Cryptococcus gatti*), and parasitic protozoa (e.g., *Plasmodium falciparum*, *Trypanosoma brucei*), suggesting clonal evolution may be widespread across several phyla (reviewed in Tibayrenc and

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Ayala 2012). But, nearly all examples are pathogens of humans or domesticated plants/animals, or are lab-reared pathogens, and these may have experienced unique selection pressures or suffer from sampling bias (Read and Day 1992). Clonal evolution in pathogens of free-ranging hosts is rarely tested or discussed (but see Morgan et al. 2007; Rajkumar et al. 2011), leaving the generality of clonal evolution among pathogens somewhat uncertain.

In some cases, clonally reproducing lineages may be similar to highly inbred and selfing species that reach a relatively late stage in the speciation process where complete, or nearly complete, reproductive isolation and the associated loss of recombination has resulted in relatively rapid and diagnosable genetic divergence between diverging lineages (Bush 1975; Templeton 1981). Because substantial genetic divergence may occur prior to the appearance of diagnosable phenotypic differences between newly speciated lineages (Barraclough et al. 2003; Sáez and Lozano 2005; Perkins et al. 2011), we expect to observe cryptic species diversity in clonally reproducing populations.

Here, we use multilocus sequence data from the lizard malaria parasite *Plasmodium floridense* to test three predictions about evolution in pathogens undergoing high rates of clonal reproduction: (1) recently diverged species will exhibit substantial genetic differentiation without evidence for diagnostic phenotypic differences (i.e., cryptic species exist); (2) putative species resulting from strongly clonal reproduction will contain low intraspecific variation; and (3) some putative species will exhibit recent interspecific divergence times. We employ a novel approach to assess PCE that centers on coalescent-based Bayesian species delimitation (Rannala and Yang 2003; Yang and Rannala 2010) to identify which, if any, lineages within *P. floridense* should be considered species. To our knowledge, this is the first explicit test of the PCE in a pathogen of free-ranging hosts.

BACKGROUND ON STUDY SYSTEM

The approximately 220 malaria parasites in the genus *Plasmodium* are an ecologically and medically important group whose life histories may be characterized by frequent clonal reproduction (Tibayrenc et al. 1990, Tibayrenc and Ayala 2002, 2012, 2014). *Plasmodium* species have an obligatory sexual life cycle where they undergo sexual reproduction in an invertebrate vector (usually a mosquito) and reproduce asexually as haploid clones in a lizard, bird, or mammal host (Perkins 2014). Obligate sexual reproduction in the vector can occur between either different or identical parasite clones (i.e., sexual or clonal reproduction, respectively), and based on evidence from the human parasites, particularly *P. falciparum*, malaria parasites are predicted to undergo predominantly clonal reproduction (i.e., the PCE; reviewed in Tibayrenc and Ayala 2012, 2014). Remember that the PCE allows for occasional sexual recombination and interbreeding among near-clades (Tibayrenc and Ayala 2012, 2014), and there is evidence of sexual recombination in populations of some otherwise clonally reproducing species (e.g., *P. falciparum*; Mzilahowa et al. 2007; Pumpaibool et al. 2009).

Plasmodium floridense is widely distributed in the Caribbean and in parts of mainland North and Central America (Fig. 1), and is among the best studied of the lizard malaria parasites (Telford 2009). Its morphology is well characterized (reviewed in Telford 2009), but it is generally indistinguishable from at least one other co-occurring species (*Plasmodium hispaniolae*; Falk et al. 2011). The mosquito *Culex erraticus* is a vector for *P. floridense* in at least part of its range (Klein et al. 1987, 1988), and both vector and parasite share a similar geographic distribution (Mendenhall et al. 2012). *Plasmodium foridense* infects only lizard vertebrate hosts, including at least 31 *Anolis* species (Squamata: Dactyloidae) and three *Sceloporus* species (Squamata: Phrynosomatidae; Telford 2009; Falk et al. 2011). It exhibits strong LD in its populations on Hispaniola (Falk et al. 2011), suggesting that it is undergoing clonal evolution.

Methods

PARASITE SAMPLING AND SEQUENCING

We sampled for *P. floridense* in lizards collected from most of the parasite's range. We collected lizards along roads and trails in Cuba (July–August 2002), Florida (March 2002, December 2002, April 2006), Hispaniola (June 2006), Jamaica (May 2012), the Puerto Rican Bank (Puerto Rico and the Virgin Islands: August 2011, October 2011), Saba (October 2005, May 2009), and Las Tuxtlas in Mexico (January 2011). From each of these lizards (except those collected in Cuba—see the following), we clipped the distal portion of one toe to obtain blood samples. One drop was used to make a thin blood smear, and we applied three to six additional drops to Whatman filter paper for molecular analysis. Blood smears for microscopic analysis were fixed in absolute methanol immediately following preparation, and were later stained with phosphate-buffered Giemsa stain for 50–60 min. We visually scanned each smear under oil immersion at 1000× magnification for 3–6 min to identify positive malaria parasite infections.

We used a molecular approach to confirm positive infections of *P. floridense*. We isolated DNA from the microscopically identified positive samples using the Qiagen DNeasy Animal Tissue Extract kit (Valencia, CA) following the manufacturer's instructions except using two elutions of 50 μl each (as opposed to 200 μl each) in the final step so that the parasite DNA would not be too diluted. Because only frozen liver samples were available from the lizards sampled in Cuba, we extracted DNA from all of these samples using the Qiagen kits (this time following the manufacturer's instructions) without first using smears to screen for malaria parasites. We used PCR of the parasite's mitochondrial

Figure 1. Putative distribution for *Plasmodium floridense*. Areas from where the parasite is previously reported are labeled, and the hypothesized distribution is shown in green.

gene cytochrome *b* (*CYTB*) to confirm malaria parasite infection in the microscopically identified positive samples, and to screen all the Cuban samples for malaria parasites. We used these sequence data and a phylogenetic approach to confirm the species identity of the parasite infections (see Supporting Information).

We sequenced eight genes from all *P. floridense* samples. These samples included each of the newly collected *P. floridense* samples and four *P. floridense* samples we previously collected on Hispaniola (Falk et al. 2011). We sequenced the same eight genes from one sample of *P. hispaniolae* (the sister taxon of *P. floridense*) to root the tree. The eight genes included two genes from the mitochondrial genome (*CYTB* and cytochrome C oxidase subunit I [*coxI*]), one gene from the apicoplast genome (caseinolytic protease C [*clpC*]), and five genes from the nuclear genome (adenylosuccinate lyase [*Adsl*], alpha-tubulin I [*Atub*], elongation factor 2 [*EF2*], histone H3 [*HisH3*], and heat shock protein 70 [*HSP70*]). We employed nested PCR with newly designed primers for all genes (primer information and thermocycler conditions are listed in the Supporting Information). Amplifications were cleaned with AMPure (Agencourt, Beverly, MA), sequenced in both directions using BigDye version 3.1 (Applied Biosystems, Foster City, CA), and edited in GENEIOUS version 5.4.6 (Biomatters, Auckland, New Zealand). Multiple sequence alignments were generated using the MUSCLE plugin (Edgar 2004) in GENEIOUS with default parameters.

TESTING PREDICTION NUMBER 1: CRYPTIC SPECIES DIVERSITY

The first prediction of the clonal-evolution hypothesis is that there is unrecognized species diversity. We employed a three-step procedure centered on Bayesian species delimitation to identify potential cryptic species diversity within *P. floridense*, where we: (1) identified putative species using both a clustering and a haplotype-identity approach; (2) inferred the relationships of the putative species using a species-tree analysis; and (3) inferred which of the putative species have their own evolutionary tendencies and historical fate (i.e., the evolutionary species concept, or ESC; Wiley 1978). This three-step procedure is a common molecular approach to species delimitation (Leaché and Fujita 2010; Burbrink et al. 2011; Leavitt et al. 2011), and the ESC is consistent with both sexually reproducing and clonally reproducing species (Wiley 1978).

As the first step in the species-delimitation pipeline, we identified putative species using discriminant analysis of principal components (DAPC; Jombart et al. 2010). DAPC is a multivariate analysis that uses sequential *K*-means clustering of principal components to identify groups of sequences, and then employs discriminant analysis to maximize variation between groups. Unlike other commonly employed methods that identify clusters of genetic data (e.g., STRUCTURE; Pritchard et al. 2000), DAPC does not optimize Hardy–Weinberg equilibrium, and so is appropriate for the haploid malaria parasite samples collected from vertebrate host populations. DAPC was run using the concatenated multiple sequence alignments of *P. floridense* samples and the ADEGENET package (Jombart 2008) in R (R Core Team 2014). We attempted to choose the optimal cluster number (*K*) of this dataset, using Bayesian information criterion (BIC) scores and each of the five available criteria (e.g., "diffNgroup" and "goodfit") in ADEGENET. The optimal *K* was variable over several runs with every criterion, however, with ≥ 10 clusters identified

in every run. For example, the "goodfit" criterion was the most stable, but inferred 11–18 clusters over 10 runs. Six clusters that generally correspond to samples collected in each area were reliably inferred over all analyses, so we chose to infer additional clusters within those six clusters using an interactive approach. We first divided the dataset into the six consistently identified sets. Next, we grouped the samples from each of these sets into one to four clusters, used discriminant analysis of just one principal component (to avoid overfitting), and chose the greatest cluster number that maximized the membership of each sample to just a single cluster.

We also identified putative species following the results of Bensch et al. (2004), where each unique *CYTB* haplotype is considered a putative species. We trimmed the *CYTB* alignment to be homologous with the region that would have amplified with the avian malaria primers HAEMF and HAEMR2 (Bensch et al. 2000). This resulted in 459-bp fragments that are slightly shorter than the 479-bp fragments that these primers amplify because we had extracted 20-bp of our primer sequences. We assigned samples to putative species based on shared haplotype identities at this locus.

As the second step in the species-delimitation pipeline, we inferred the relationships of the putative species using a species-tree analysis in *BEAST version 1.6.2 (Drummond and Rambaut 2007; Heled and Drummond 2010). *BEAST is a Bayesian Markov chain Monte Carlo (MCMC) method that uses a multilocus coalescent model to estimate the species tree. We used the putative species identified via DAPC because this method resulted in a greater number of putative species than were identified based on *CYTB* haplotype identity (see Results), which allowed us to test among more species hypotheses. We gave each putative species an arbitrary name according to which the samples were collected (e.g., "Hispaniola 1"). We chose substitution models for each locus using BIC scores in jModelTest version 0.1.1 (Posada 2008). In loci for which clock-like evolution was rejected using likelihood ratio tests in MEGA version 5.05 (Tamura et al. 2011), we used a relaxed clock with a lognormal prior because these priors are robust to misspecification (Wertheim et al. 2010). We used strict clocks for loci for which we could not reject clock-like evolution. The final analysis was set to run for 6.0×10^8 generations, sampled every 6.0×10^4 generations, with the first 10% discarded as burn-in. We assessed convergence in TRACER by checking that the effective sample size (ESS) values for every parameter were ≥200, and that the posteriors had unimodal distributions that pulled away from the priors.

In the third and final step in the species-delimitation pipeline, we estimated the probability that putative species are independently evolving using the Bayesian species delimitation program BPP version 2.1 (Rannala and Yang 2003; Yang and Rannala 2010). BPP employs a reversible-jump MCMC to estimate the probability of alternative species delimitation models, conditioned on the probabilities of population size and time since divergence among species in the various possible delimitations (i.e., it assumes that sequences from the same species will produce higher probability estimates of population size and species divergence than will sequences from different species). Incomplete lineage sorting is accommodated via a coalescent model. The program has been used to successfully delimit both asexually and sexually reproducing species (Yang and Rannala 2010; Fujita et al. 2012), and is consistent with the ESC, and so is appropriate for delimitation of potentially clonally reproducing species. This BPP algorithm samples a user-defined, bifurcating guide tree of putative species, and whereas it can collapse and resolve previously collapsed nodes, it cannot move branches on the tree or incorporate phylogenetic uncertainty into its estimations (but note that a newer BPP algorithm allows simultaneous inference of species limits and relationships; Yang and Rannala 2014). Accordingly, we took a conservative approach for our guide tree and collapsed any terminal nodes on the *BEAST species tree with <95% posterior probability. Following Burbrink et al. (2011), we employed BPP algorithm 0 with multiple values of the fine-tuning parameter ε (5, 10, 20) to ensure adequate performance of the rjMCMC. Prior distributions for ancestral population mutation rate (θ) and root age (τ_0) may affect the posterior probabilities of the species models (Yang and Rannala 2010), and so we used three different—but still diffuse—prior combinations to evaluate the sensitivity of our dataset to these priors (Table 1; Leaché and Fujita 2010). We parameterized our model to accommodate rate variation among loci, imposing a Dirichlet prior distribution with vector $\alpha = 2$. We also used $\alpha = 10$, which corresponds to greater variation among loci, over all prior combinations for $\varepsilon = 10$ to check the sensitivity of our dataset to this prior. Each analysis was run for 150,000 generations and sampled every three generations, with the first 10% discarded as burn-in. We adjusted the step proposals of the fine-tune parameters and allowed the program to automatically adjust these during burn-in, and these were satisfactory for all analyses, remaining in the interval (0.2, 0.6). Each analysis was run twice with different starting seeds to confirm consistency among runs, and we checked that the ESS values of all parameters in all runs were ≥200. We considered putative species to be independently evolving when the posterior probabilities were ≥95% in all nine parameterizations (each of three values of the fine-tuning parameter ε under each of the three prior combinations).

TESTING PREDICTION NUMBER 2: LOW VARIATION WITHIN SPECIES

The second prediction of the clonal-evolution hypothesis is that intraspecific genetic variation is low, and we estimated two population genetics parameters of the BPP-identified species to assess intraspecific variation. For each species separately and all

Table 1. Prior probabilities in the BPP analyses.

	θ		τ_0	
Prior (description)	Γ (α, β)	$m = s$	Γ (α, β)	$m = s$
Prior 1 (small θ, small τ_0)	Γ (1, 5000)	2.0×10^{-4}	Γ (1, 5000)	2.0×10^{-4}
Prior 2 (large θ, large τ_0)	Γ (1, 10)	1.0×10^{-1}	Γ (1, 10)	1.0×10^{-1}
Prior 3 (large θ, small τ_0)	Γ (1, 10)	1.0×10^{-1}	Γ (1, 5000)	2.0×10^{-4}

BPP analyses were run under three different prior combinations for ancestral population mutation rate (θ) and root age (τ_0). These priors are assigned a gamma (Γ) distribution (α, β), with a mean $m = \alpha/\beta$ and standard deviation $s = (\alpha/\beta^2)^{1/2}$.

combined, we used DnaSP version 5 (Librado and Rozas 2009) to measure the number of haplotypes in each locus (N_h) and nucleotide diversity (π). Our expectation is that clonally reproducing species will be characterized by just one haplotype per locus, and when there is more than one haplotype per locus, nucleotide diversity will be low (e.g., <0.005).

We also estimated θ for each species as an alternative measure of intraspecific genetic variation. This parameter is the product of the effective population size N_e, the per-generation mutation rate μ, and the appropriate ploidy scalar, and unlike similar estimators (e.g., direct estimates of N_e), it does not make any assumptions about μ. We estimated θ of each BPP-inferred species in two steps. First, we inferred a phylogeny of the BPP-inferred species, using *BEAST with the same parameterizations, priors, and convergence diagnostics as described for the guide tree phylogeny. Second, we estimated θ of each species using this species tree and the package BIOPY version 0.1.7 (Heled 2011) in Python version 2.7 (http://www.python.org). We expect clonally reproducing species to exhibit low θ estimates (e.g., <0.01).

TESTING PREDICTION NUMBER 3: RECENT DIVERGENCE BETWEEN SPECIES PAIRS

The third prediction of the clonal-evolution hypothesis is that speciation occurs over short timescales and so some species pairs would have recently diverged, and we employed a species-tree approach in *BEAST to infer an ultrametric, dated phylogeny of the BPP-identified species to test the prediction of recent divergence. We time-calibrated our phylogeny using a molecular clock rate for malaria parasites as estimated by Ricklefs and Outlaw (2010). They compared a phylogeny of malaria parasites to a phylogeny of their avian hosts, and used the proportional differences between host sister taxa and parasite sister taxa, conditioned on the relative age of host-switching events and the molecular clock rate of birds, to estimate a mean parasite per-lineage *CYTB* rate of 0.6% per million years. This clock-rate estimate produces divergence date estimates for primate malaria parasites that are roughly congruous with estimates that use host fossil and biogeographic calibrations (Pacheco et al. 2011). But, whereas the 0.6% rate is lower than the rates commonly employed in arthropods (1.0–1.2%; Brower 1994) and vertebrates (approximately 1.0%; Bromham 2002; Weir and Schluter 2008), it includes assumptions that may cause it to be artificially high (Bensch et al. 2013). Nonetheless, we lack suitable alternative calibrations (e.g., biogeographic or host constraints; see Supporting Information), and so used this *CYTB* rate and the same *BEAST parameterizations, priors, and convergence diagnostics as in the abovementioned guide-tree inference to infer a time-calibrated phylogeny of *P. floridense* species. We consider divergence times of <0.5 million years ago (MYA) to be recent and consistent with our prediction.

As an alternative to the dating estimates, we quantified the difference between the most recently diverged species pairs, expecting the difference to be minimal because of recent divergence. We used DnaSP and the concatenated dataset to measure the total number of fixed sites and the average number of nucleotide substitutions per site (D_{xy}) between species using a Jukes–Cantor substitution model. We consider D_{xy} values of <1% and fixed substitutions of <30 (across the concatenated dataset) to represent minimal interspecific divergence.

Results

PARASITE SAMPLING AND SEQUENCING

We successfully sequenced all eight genes from 63 *P. floridense* samples, which included 59 newly identified, single-genotype infections, and four samples we previously collected and identified on Hispaniola (Falk et al. 2011; see Supporting Information). We also sequenced all eight genes from one *P. hispaniolae* sample, resulting in 4202-bp of data with no missing data or gaps. Summary information for these genes (including sequence lengths and best-fit substitution models) and samples (including sampling localities and host species) are provided in the Supporting Information.

TESTING PREDICTION NUMBER 1: CRYPTIC SPECIES DIVERSITY

We identified nearly twice as many putative *P. floridense* species with DAPC than with *CYTB* haplotypes. We inferred a total of 17 genetic clusters using DAPC (Fig. 2A), and recovered nine unique haplotypes of 459-bp *CYTB* (Fig. 2B). In four instances,

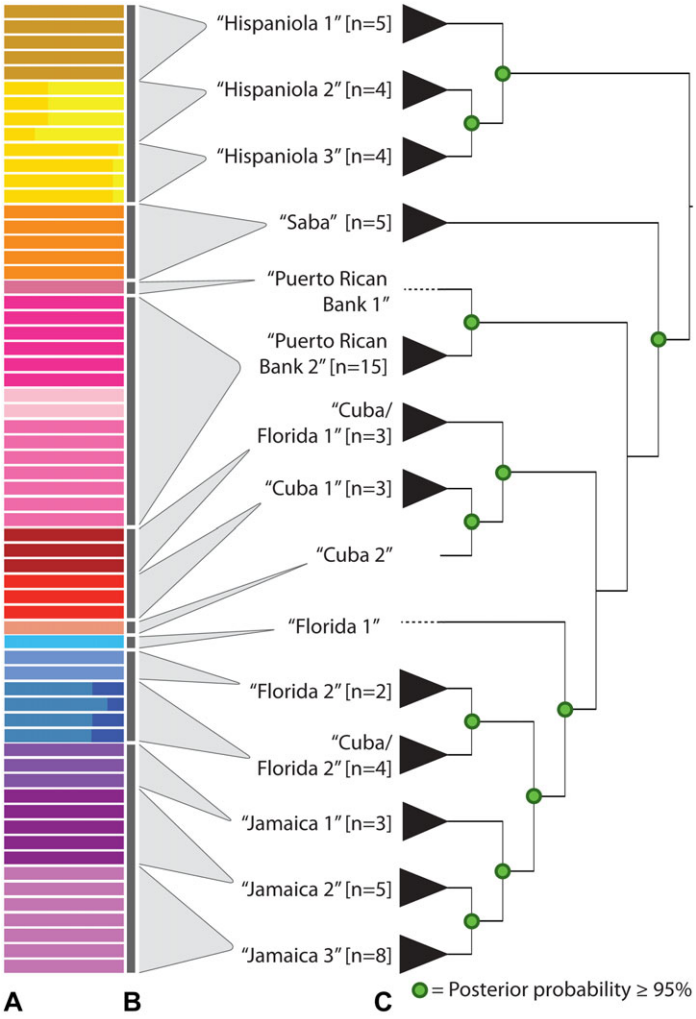

Figure 2. **Putative species identification and *BEAST guide tree. (A) Results of DAPC showing the 17 identified putative species. Each bar represents a single sample and color represents inferred species identity. (B) Nine haplotypes (459-bp *CYTB*) are shared among samples, as indicated by each continuous gray bar. (C) Guide tree of 15 putative lineages for BPP analyses, inferred using a species tree approach in *BEAST. Each of the 17 DAPC putative species was initially included, but two unsupported terminal nodes were collapsed until posterior probabilities reached 95%. All but two internal nodes have ≥95% posterior probability, and all terminal nodes have ≥95% posterior probability.**

the DAPC and *CYTB*-haplotype inferences were congruent, and the remaining 13 DAPC clusters were contained within five *CYTB* haplotypes.

We inferred a guide tree of the DAPC-inferred putative species using *BEAST (Fig. 2C). There were four DAPC-inferred species on the Puerto Rican Bank, and the relationships among three of these—corresponding to samples collected on Puerto Rico, St. Thomas/St. John, and Virgin Gorda—are unresolved. We collapsed these nodes for the BPP guide tree. Except for two nodes near the base of the tree, all the remaining nodes are well supported with ≥95% posterior probability.

Of the 15 putative species within *P. floridense*, 11 were recovered as being evolutionary independent lineages (i.e., species) in each of the nine BPP analysis run under three different prior combinations and three different values for fine-tune parameter ε (Fig. 3). In all analyses, "Hispaniola 2" and "Hispaniola 3" are collapsed into a single species, as are "Cuba/Florida 2" and "Florida 2." In six of the nine analyses, "Cuba 1," and "Cuba 2" are collapsed into a single species, as are "Jamaica 2" and "Jamaica 3." These results were consistent between the two independent runs, and were unchanged in analyses using $\alpha = 10$ instead of $\alpha = 2$ for the Dirichlet prior on locus rate variation.

TESTING PREDICTION NUMBER 2: LOW VARIATION WITHIN SPECIES

Genetic variation within each of the BPP-inferred *P. floridense* species is low. The number of haplotypes and nucleotide

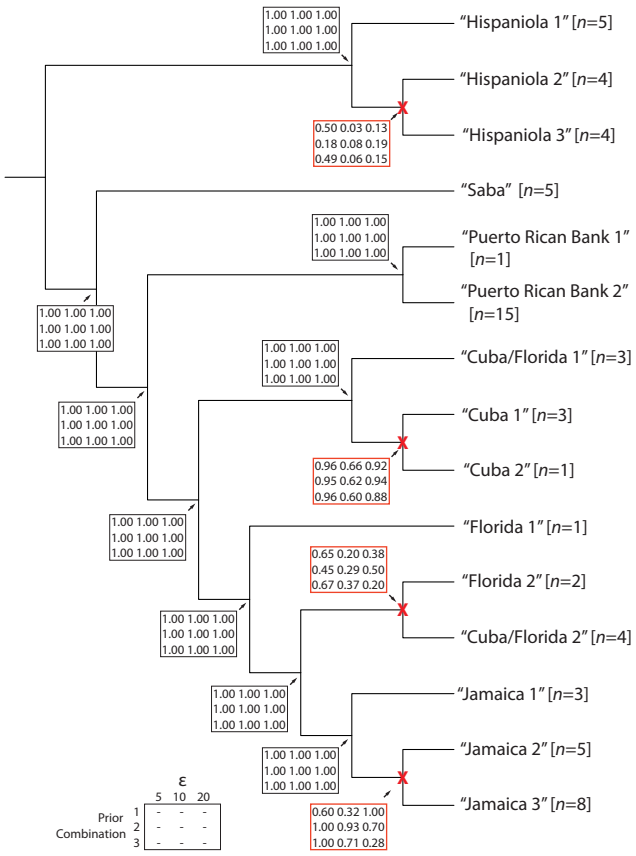

Figure 3. Posterior probabilities of reproductive isolation between species, as inferred from nine BPP analyses. Node labels represent the probability that descendent species are reproductively isolated, given the model parameters and priors. Of the 15 putative species, 11 are inferred to be reproductively isolated in every analysis.

diversity are shown in Table 2. We observed just 30 unique 4202-bp sequences among all 11 species and 63 samples of *P. floridense*. Most species contained just one haplotype at each locus (e.g., all species except "Puerto Rican Bank 2" possess just one haplotype at *clpC*). Likewise, when a species contains more than one haplotype per locus, nucleotide diversity is low and ranges 0.00016–0.00375. All θ estimates were low, with mean estimates ranging 0.001–0.002 (Fig. 4A).

TESTING PREDICTION NUMBER 3: RECENT DIVERGENCE BETWEEN SPECIES PAIRS

We observed recent divergence estimates between species. Using the molecular clock rate in *BEAST, we estimated a crown age of our samples at 0.89 MYA (95% CI: 0.58–1.2 MYA) (Fig. 4B). Divergence dates and summary statistics of the four most recently diverged species pairs are shown in Table 3. These species pairs, diverged approximately 0.11–0.27 MYA (95% CI: 0.038–0.41 MYA), have just six to 15 fixed sites between species within the 4202-bp concatenated alignments, and differ by just approximately 0.2–0.4%.

Discussion

We showed that diversification in *P. floridense* is strongly impacted by clonal reproduction, and identified 11 provisional species contained within *P. floridense* that exhibit low within-species variation and recent divergence between species. Our results suggest that at least in some cases, high rates of clonal reproduction can affect pathogen evolution beyond previous predictions of LD and near-clading, and ultimately lead to complete genetic isolation and speciation. Our results also suggest that clonal evolution affects pathogens of free-ranging wildlife, and that it is not simply a result of human-associated evolution.

CRYPTIC MALARIA PARASITE SPECIES

Our analyses of DNA sequence data in a Bayesian species delimitation pipeline resulted in the identification of 11 putatively cryptic species within one previously recognized and relatively well-characterized species. Although the generality of this discovery requires analyses of additional taxa, our results suggest that the number of undescribed pathogen species may be enormous. Morphological crypsis among malaria parasites has been previously documented (Perkins 2000; Martinsen et al. 2006; Sehgal et al. 2006; Falk et al. 2011; Palinauskas et al., in press), but few studies have provided evidence of a diversity underestimation of a similar magnitude as ours. Bensch et al. (2004) is among those few, and used multilocus data to show that there may be as many *Plasmodium* species as they have 479-bp *CYTB* haplotypes. We show that the one-CYTB-haplotype-per-malaria-parasite-species estimate may be conservative in some cases, as we used DAPC and Bayesian species delimitation to infer two more species than the nine 459-bp *CYTB* haplotypes (but note that some *P. floridense* species had more than one *CYTB* haplotype). Similarly, a 3–5% genetic distance threshold between *CYTB* sequences has been proposed to distinguish between putative malaria parasite species (reviewed in Outlaw and Ricklefs 2014). Intraspecific mtDNA divergence within *P. floridense* species ranged 0–0.4% (Table 2), but interspecific sequence divergence of the four most recently diverged species similarly ranged 0.2–0.4% (Table 3), suggesting that any similarity threshold may be either very conservative or potentially misleading.

Rather than describe the 11 *P. floridense* species at this time, we hope to initiate dialogue among systematists about the taxonomic issues associated with clonally reproducing species, and to move forward with consensus. A desirable quality of the classification of these species is that species delimitation be objective and repeatable, and philosophically grounded in biology. In the context of our study, it is important to ask whether the species we

Table 2. Number of samples (*N*), number of haplotypes (N_h), and nucleotide diversity (π) in the *Plasmodium floridense* species.

Locus	Lineage	*N*	N_h	π	Locus	Lineage	*N*	N_h	π
Adsl	All	63	18	0.02393	*EF2*	All	63	10	0.00646
	Cuba 1, 2	4	1	0		Cuba 1, 2	4	3	0.00285
	Cuba/Florida 1	3	1	0		Cuba/Florida 1	3	1	0
	Cuba/Florida 2, Florida 2	6	4	0.00218		Cuba/Florida 2, Florida 2	6	1	0
	Florida 1	1	1	0		Florida 1	1	1	0
	Hispaniola 1	5	3	0.00138		Hispaniola 1	5	1	0
	Hispaniola 2, 3	8	2	0.00043		Hispaniola 2, 3	8	2	0.00153
	Jamaica 1	3	1	0		Jamaica 1	3	1	0
	Jamaica 2, 3	12	1	0		Jamaica 2, 3	12	1	0
	Puerto Rican Bank 1	1	1	0		Puerto Rican Bank 1	1	1	0
	Puerto Rican Bank 2	15	2	0.00043		Puerto Rican Bank 2	15	1	0
	Saba	5	1	0		Saba	5	1	0
Atub	All	63	13	0.01165	*HisH3*	All	63	6	0.00410
	Cuba 1, 2	4	2	0.00092		Cuba 1, 2	4	2	0.00164
	Cuba/Florida 1	3	2	0.00016		Cuba/Florida 1	3	1	0
	Cuba/Florida 2, Florida 2	6	3	0.00123		Cuba/Florida 2, Florida 2	6	2	0.00219
	Florida 1	1	1	0		Florida 1	1	1	0
	Hispaniola 1	5	2	0.00074		Hispaniola 1	5	1	0
	Hispaniola 2, 3	8	2	0.00046		Hispaniola 2, 3	8	1	0
	Jamaica 1	3	1	0		Jamaica 1	3	1	0
	Jamaica 2, 3	12	1	0		Jamaica 2, 3	12	1	0
	Puerto Rican Bank 1	1	1	0		Puerto Rican Bank 1	1	1	0
	Puerto Rican Bank 2	15	1	0		Puerto Rican Bank 2	15	1	0
	Saba	5	1	0		Saba	5	1	0
clpC	All	63	10	0.00703	*HSP70*	All	63	8	0.00540
	Cuba 1, 2	4	1	0		Cuba 1, 2	4	2	0.00375
	Cuba/Florida 1	3	1	0		Cuba/Florida 1	3	1	0
	Cuba/Florida 2, Florida 2	6	1	0		Cuba/Florida 2, Florida 2	6	1	0
	Florida 1	1	1	0		Florida 1	1	1	0
	Hispaniola 1	5	1	0		Hispaniola 1	5	1	0
	Hispaniola 2, 3	8	1	0		Hispaniola 2, 3	8	2	0.00094
	Jamaica 1	3	1	0		Jamaica 1	3	1	0
	Jamaica 2, 3	12	1	0		Jamaica 2, 3	12	1	0
	Puerto Rican Bank 1	1	1	0		Puerto Rican Bank 1	1	1	0
	Puerto Rican Bank 2	15	2	0.00245		Puerto Rican Bank 2	15	1	0
	Saba	5	1	0		Saba	5	1	0
mtDNA	All	63	16	0.00813	Complete	All	63	30	0.01005
(*coxI* and	Cuba 1, 2	4	1	0	Dataset	Cuba 1, 2	4	4	0.00071
CYTB)	Cuba/Florida 1	3	2	0.0038		Cuba/Florida 1	3	2	0.00016
	Cuba/Florida 2, Florida 2	6	3	0.00057		Cuba/Florida 2, Florida 2	6	6	0.00086
	Florida 1	1	1	0		Florida 1	1	1	0
	Hispaniola 1	5	1	0		Hispaniola 1	5	4	0.00029
	Hispaniola 2, 3	8	1	0		Hispaniola 2, 3	8	4	0.00031
	Jamaica 1	3	1	0		Jamaica 1	3	1	0
	Jamaica 2, 3	12	3	0.00040		Jamaica 2, 3	12	3	0.00017
	Puerto Rican Bank 1	1	1	0		Puerto Rican Bank 1	1	1	0
	Puerto Rican Bank 2	15	1	0		Puerto Rican Bank 2	15	3	0.00030
	Saba	5	1	0		Saba	5	1	0

We observed just one haplotype per locus in most species, and when more than one haplotype is present, nucleotide diversity is very low and ranges 0.00016–0.00375.

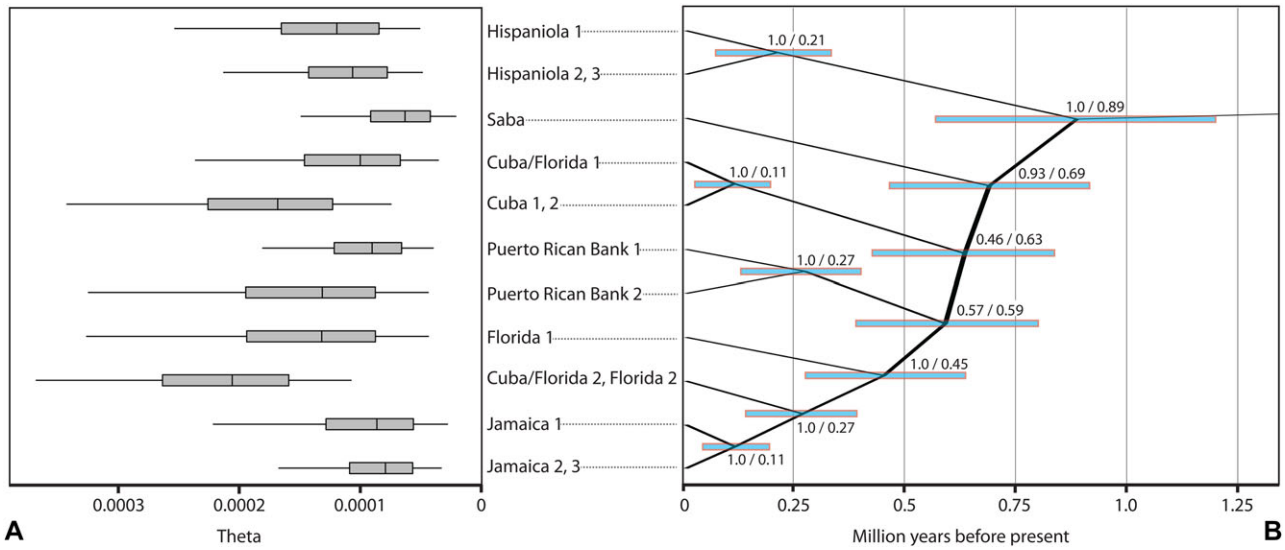

Figure 4. θ and divergence date estimates for the 11 *Plasmodium floridense* species. (A) θ estimates were estimated from a *BEAST species tree using BIOPY. The box plots show the mean, standard deviation, and 95% CI for each estimate. Mean θ estimates are very low at <0.0003. (B) A chronogram was inferred using a species-tree approach in *BEAST and a molecular clock rate for malaria parasites. Nodes are labeled with posterior probability/mean divergence time, and node bars indicate the 95% CI for the age of that node. All species diverged in approximately the last million years.

Table 3. Differentiation among the four most closely related sister species.

Species X	Species Y	Fixed Differences	d_{xy}	tMRCA
Cuba 1, 2	Cuba/Florida 1	6	0.211%	0.1101 MYA
			(0.083%)	[0.0382–0.2009]
Hispaniola 1	Hispaniola 2, 3	12	0.357%	0.2080 MYA
			(0.102%)	[0.0951–0.3510]
Jamaica 1	Jamaica 2, 3	8	0.203%	0.1117 MYA
			(0.087%)	[00447–0.1931]
Puerto Rico 1	Puerto Rico 2	15	0.380%	0.2695 MYA
			(0.230%)	[0.1357–0.4055]

We report the number of fixed differences between species in the 4202 bp dataset, the number of nucleotide substitutions per site between species (d_{xy}; with standard deviation shown in parentheses), and time to most recent common ancestor (tMRCA; with 95% CI in parentheses).

delimited should be considered species, and we discuss this question in terms of both our analytic approach and species concept.

We contend that our analytical approach to species delimitation was suitable. We delimited the 11 species using seven separately evolving loci and a Bayesian species delimitation algorithm that has been used in many kinds of taxa, including malaria parasites (Ramiro et al. 2012) and asexual fungi (Yang and Rannala 2010). The basic assumption of the BPP algorithm is that members of each species share the same population-genetic parameters of population size and time since divergence, but perhaps near-clades could have the same result? A simulation study showed that the BPP algorithm is sensitive to low-to-moderate levels of gene flow, and will not consider species to be independently evolving once interspecific gene flow reaches 0.1–10 migrants per generation (Zhang et al. 2011). This suggests that at some level of recombination among near-clades, BPP would combine the near-clades into a single species, and this may be why BPP rejected some of the putative species hypotheses we tested.

The species we delimited may or may not be philosophically sound. Clonally reproducing lineages are considered species under the species concept we employed (the ESC; Wiley 1978), as well as others. For example, the phylogenetic species concept (Cracraft 1983) has been successfully applied to malaria parasites (Perkins 2000; Martinsen et al. 2006) and asexually reproducing organisms, such as fungi (Taylor et al. 2000). Clonally reproducing lineages are not considered species under some species concepts, however. The general lineage concept (GLC), for instance, defines a species as an interbreeding, metapopulation lineage (de Queiroz 1998, 2007), and because clonally reproducing organisms do not interbreed they are not considered species under

this concept. But, clonally reproducing organisms may be unified as species under the GLC by other shared features (e.g., natural selection; de Queiroz 2005).

We did not collect morphological data from the 11 *P. floridense* species, so the species we delimited are only provisionally cryptic and it is possible that there are morphological characters to distinguish among them. Morphology is generally not informative for this group, however, as parasite morphology changes according to host species, and morphological characters are few (Falk et al. 2011; Perkins et al. 2011). *Plasmodium floridense* is morphologically cryptic with *P. hispaniolae*, for example (Falk et al. 2011). Also, the sample we collected in Mexico (where *P. floridense* was previously reported; Lowichik et al. 1988) was identified as *P. fairchildi* using a molecular phylogenetic approach, even though it exhibited morphology consistent with *P. floridense* (i.e., *P. floridense* and *P. fairchildi* are also morphologically cryptic; see Supporting Information).

Nonetheless, morphological data are not necessary for a species description, as nontraditional species descriptions that lack morphology are compliant with the rules in the International Commission on Zoological Nomenclature (i.e., the ICZN, or The Code) under certain conditions. The use of molecular characters—in lieu of morphological characters—for species descriptions is compliant with The Code (Article 13.1.1; ICZN 1999). Type material may be assigned using either the traditional approach of a blood or tissue preparation containing life stages of the parasite (i.e., a hapantotype; Article 73.3; ICZN 1999), or a nontraditional approach where tissues from which the parasite's DNA was amplified are designated as types (because blood or tissue preparations are not available). This nontraditional approach is also Code-compliant, as "the type can be the specimen or part of the specimen, for example, a tissue sample, or pre-amplification DNA sample, on which the sequence is based (Article 72.5.6)" (http://iczn.org/node/814 [accessed June 4, 2014]). A molecular-only approach to species identification and delimitation is not free of potential problems (e.g., mixed infections and PCR bias; Valkiūnas et al. 2006), but we believe those problems will be ameliorated as molecular data become easier and less expensive to generate.

LOW VARIATION WITHIN SPECIES

We observed very low genetic variation in the *P. floridense* species, although the extent of variation was variable among species. Some of this variability may be attributable to geographic area. For example, all five samples collected on Saba, which is a very small island (13km^2), were genetically identical (this was the only species for which we had multiple samples that were identical at all loci). Similarly, genetic diversity was highest in a species with samples collected from different areas, "Cuba/Florida 2, Florida 2" (Table 3; Fig. 4A), suggesting that increased intraspecific diversity may be caused by dispersal into new areas, with subsequent isolation and divergence. Significant gene flow between populations on Cuba and Florida is unlikely, and we expect these populations will continue to diverge and eventually speciate. If true, then genetic diversity in clonally reproducing species may oscillate as populations disperse (genetic diversity increases in parent species) and then complete speciation (genetic diversity decreases in each daughter species).

RECENT DIVERGENCE BETWEEN SPECIES PAIRS

We observed very recent divergence date estimates between *P. floridense* species, with a mean estimate of approximately 0.9 MYA for all 11 species, and the most recent divergence estimates among species pairs at approximately 0.1 MYA. In one instance, two species were sampled at the same locality ("Cuba/Florida 1" and "Cuba/Florida 1, Florida 2" in Florida—see Supporting Information), and these show no evidence of sexual recombination. These estimates are much more recent than the most recent divergence estimates of *Anolis* species (e.g., *Anolis desechensis* approximately 1.3 MYA, Brandley and de Queiroz 2004; *Anolis fuscoauratus* approximately 3 MYA, Glor et al. 2001), indicating that the relationships between the extant *P. floridense* species and their vertebrate hosts are relatively new. For the species tested, virulence of *P. floridense* species in their host species is low (Schall and Pearson 2000; Schall and Staats 2002), which seems to present yet another example in contrast of the old dogma that parasites evolve lower virulence over time (i.e., the "trade-off" hypothesis; Anderson and May 1982; Alizon et al. 2009). The recent divergence also indicates that any biogeographic patterns in the *P. floridense* species are due to recent events, and any cophylogenetic patterns will be temporally incongruent with the vertebrate hosts at the species level.

WHAT DETERMINES THE RATE OF CLONAL REPRODUCTION?

The high rates of clonal evolution in *P. floridense* may be associated with low prevalence, because sexual recombination is more likely when a vector ingests a blood meal containing more than one parasite genotype, and the proportion of vertebrate hosts infected with more than one parasite genotype increases as prevalence of each of those genotypes increases. Prevalence of *P. floridense* in its vertebrate hosts is generally low (e.g., $<5\%$ in a survey of almost 700 lizards on Hispaniola; Falk et al. 2011). Also, we did not observe any infections mixed with more than one *P. floridense* species or haplotype of the same *P. floridense* species in our dataset, even when different species occurred in the same area (samples FL509 and FL513 each belong to different species but were collected at the same locality; see Supporting Information). PCR bias can cause similar results (i.e., inference of a single infection when multiple infections are present; Valkiūnas et al.

2006), but we did observe two samples that were mixed between *P. floridense* and other species (e.g., *P. hispaniolae*; see Supporting Information), suggesting that mixed infections are detectable when using our protocols.

In addition to dispersal and subsequent speciation as described above, geographic variation in prevalence of the *P. floridense* species may be associated with speciation. *Plasmodium floridense* is not continuously distributed across the landscape, but exhibits a "patchy" distribution where hosts at most local host populations are not infected (i.e., local parasite populations are geographically isolated from each other). On Hispaniola, *P. floridense* was observed in just 15 of 52 sampled localities, despite an abundance of available vertebrate hosts (Falk et al. 2011). This patchy prevalence, and the fact that only one *P. floridense* species was sampled at any given locality (with one exception, mentioned above), suggests that geographic isolation may lead to speciation. There is no evidence that host switching causes speciation in *P. floridense*. Multiple *P. floridense* species infect the same vertebrate host species when occurring on the same island (e.g., both Hispaniolan species infect *Anolis cybotes*; see Supporting Information). *Culex erraticus* is a competent vector for *P. floridense* (Klein et al. 1987, 1988), but if other mosquito species also vector the parasite (this has not been well explored), then speciation events in *P. floridense* may be precipitated by changes in vector species.

Understanding the causes of differences in prevalence may be the key to understanding clonal reproduction—and possibly speciation—for *P. floridense* and other pathogens where sexual recombination occurs in a vector. We already have some knowledge of the factors contributing to differences in pathogen prevalence in wild host populations. For example, certain environmental variables (e.g., availability of water, temperature) are associated with differences in prevalence in *P. floridense* (Perkins et al. 2007), as well as avian trypanosome parasites (Sehgal et al. 2011) and malaria parasites (Wood et al. 2007; Sehgal et al. 2011). Year-round transmission, as opposed to seasonal transmission, is associated with higher prevalence rates among avian malaria parasites (Pérez-Tris and Bensch 2005). Similarly, generalist malaria parasites reach higher prevalence rates in their avian hosts than do parasites specializing on fewer species (Hellgren et al. 2009). Whether these traits and conditions are associated with differences in clonal reproduction remains untested.

Differences in virulence may also play a role in determining the extent of clonal reproduction in pathogens when more than one clone is present in a local host population (Mackinnon and Read 1999). If one clone is more virulent than another, reaching higher infection intensity in the same host individual, then the more virulent clone is more likely to be taken up by the vector (i.e., rates of clonal reproduction may increase as differences in virulence among clones increase; Schall 2000). Variation in infection intensity may be more strongly associated with host effects rather than pathogen genotype (Neal and Schall 2014), suggesting a mechanism in which characteristics of the host may affect reproduction in the pathogen. In any case, our results suggest that clonal reproduction may be widespread among pathogens generally, and further research into the factors that influence the extent of clonal reproduction may shed light on the evolution on a great number of organisms.

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DATA ARCHIVING

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Phylogeny used for malaria parasite identification.
Table S1. GenBank accession numbers and collecting localities for samples included in the identification phylogeny
Table S2. Primer information & PCR conditions
Table S3. Host & locality information
Table S4. Summary information for each locus